University of Groningen

Comparative proteomic analysis of a potentially probiotic *Lactobacillus pentosus* MP-10 for the identification of key proteins involved in antibiotic resistance and biocide tolerance

Casado Muñoz, María del Carmen; Benomar, Nabil; Ennahar, Saïd; Horvatovich, Peter; Lavilla Lerma, Leyre; Knapp, Charles W.; Gálvez, Antonio; Abriouel, Hikmate

Published in:
International Journal of Food Microbiology

DOI:
[10.1016/j.ijfoodmicro.2016.01.012](https://doi.org/10.1016/j.ijfoodmicro.2016.01.012)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Casado Muñoz, M. D. C., Benomar, N., Ennahar, S., Horvatovich, P., Lavilla Lerma, L., Knapp, C. W., Gálvez, A., & Abriouel, H. (2016). Comparative proteomic analysis of a potentially probiotic *Lactobacillus pentosus* MP-10 for the identification of key proteins involved in antibiotic resistance and biocide tolerance. *International Journal of Food Microbiology*, 222, 8-15. <https://doi.org/10.1016/j.ijfoodmicro.2016.01.012>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Comparative proteomic analysis of a potentially probiotic *Lactobacillus pentosus* MP-10 for the identification of key proteins involved in antibiotic resistance and biocide tolerance

María del Carmen Casado Muñoz^a, Nabil Benomar^a, Saïd Ennahar^b, Peter Horvatovich^c, Leyre Lavilla Lerma^a, Charles W. Knapp^d, Antonio Gálvez^a, Hikmate Abriouel^{a,*}

^a Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Universidad de Jaén, 23071 Jaén, Spain

^b Equipe de Chimie Analytique des Molécules Bio-Actives, UMR 7178, IPHC-DSA, Université de Strasbourg, CNRS, 67400 Illkirch-Graffenstaden, France

^c Department of Analytical Biochemistry, Centre for Pharmacy, University of Groningen, Groningen, The Netherlands

^d Department of Civil and Environmental Engineering, University of Strathclyde, Glasgow, Scotland, United Kingdom

ARTICLE INFO

Article history:

Received 27 June 2015

Received in revised form 10 December 2015

Accepted 20 January 2016

Available online 22 January 2016

Keywords:

Comparative proteomics

Lactobacillus pentosus

Probiotics

Antibiotics

Biocides

ABSTRACT

Probiotic bacterial cultures require resistance mechanisms to avoid stress-related responses under challenging environmental conditions; however, understanding these traits is required to discern their utility in fermentative food preparations, versus clinical and agricultural risk. Here, we compared the proteomic responses of *Lactobacillus pentosus* MP-10, a potentially probiotic lactic acid bacteria isolated from brines of naturally fermented Aloreña green table olives, exposed to sub-lethal concentrations of antibiotics (amoxicillin, chloramphenicol and tetracycline) and biocides (benzalkonium chloride and triclosan). Several genes became differentially expressed depending on antimicrobial exposure, such as the up-regulation of protein synthesis, and the down-regulation of carbohydrate metabolism and energy production. The antimicrobials appeared to have altered *Lb. pentosus* MP-10 physiology to achieve a gain of cellular energy for survival. For example, biocide-adapted *Lb. pentosus* MP-10 exhibited a down-regulated phosphocarrier protein HPr and an unexpressed oxidoreductase. However, protein synthesis was over-expressed in antibiotic- and biocide-adapted cells (ribosomal proteins and glutamyl-tRNA synthetase), possibly to compensate for damaged proteins targeted by antimicrobials. Furthermore, stress proteins, such as NADH peroxidase (Npx) and a small heat shock protein, were only over-expressed in antibiotic-adapted *Lb. pentosus* MP-10. Results showed that adaptation to sub-lethal concentrations of antimicrobials could be a good way to achieve desirable robustness of the probiotic *Lb. pentosus* MP-10 to various environmental and gastrointestinal conditions (e.g., acid and bile stresses).

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Lactobacillus pentosus is the most prevalent species of lactic acid bacteria (LAB) found in naturally-fermented Aloreña table olives (Abriouel et al., 2011, 2012) and Spanish-style green fermented olives (Maldonado-Barragán et al., 2011). Furthermore, these versatile bacteria have been detected in various environmental niches such as plant materials, silage, fermented foods (dairy, vegetable and meat), as well as the oral cavities, gastrointestinal tracts (GIT), and vaginas of humans and animals (Anukam et al., 2013; Okada et al., 1986; Tajabadi et al., 2011; Todorov and Dicks, 2004). Due to their wide distribution and beneficial effects, special and deserved attention was recently given to the application of lactobacilli, especially of vegetable origin, as a starter

culture in different fermentations (Rodríguez-Gómez et al., 2014; Ruiz-Barba and Jiménez-Díaz, 2012), as a probiotic in silage (EFSA, 2011), dairy (Anukam and Olise, 2012) and fermented olives (Rodríguez-Gómez et al., 2014), as they provide bio-therapeutic benefits via bacterial pathogen inhibition and an improved immune system. More specifically, *Lb. pentosus* MP-10 isolated from brine of naturally fermented Aloreña olives (Abriouel et al., 2011, 2012) could be used as a probiotic strain due to their ability to inhibit pathogenic bacteria and tolerate low pH (1.5) and bile salts (3%) in the gastrointestinal environment.

Besides the technological and health-promoting effects shown by lactobacilli with probiotic properties, such as production of antimicrobial substances and survival in gastrointestinal tracts, other requirements should be proven to justify their utility. The most important selection criteria for bacterial strains intended for use as probiotics include: 1) intrinsic resistance to antibiotics of human and veterinary importance and 2) lack of transferable resistance genes to avoid the risk of horizontal gene transfer to other bacteria in the food chain and environment

* Corresponding author at: Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Edif. B3, Universidad de Jaén, Campus Las Lagunillas s/n, 23071 Jaén, Spain.

E-mail address: hikmate@ujaen.es (H. Abriouel).

(EFSA, 2008; EFSA Panel on Biological Hazards, 2010). As such, many studies have focused on genotypic methods to highlight the presence or absence of antimicrobial resistance determinants (e.g., Bautista-Gallego et al., 2013; Duran and Marshall, 2005; EFSA, 2012; Franz et al., 1999; Zhang et al., 2009). However, several aspects of bacterial fitness, which develop tolerance or resistance to different antimicrobials used in a clinical setting or disinfection, remain unexplored. Bacterial adaptation to antimicrobials, which was referred by Maisonneuve and Gerdes (2014) as “bacterial persisters,” is the intermediary stage that links between sensitive and resistant phenotypes. Thus, more attention should be provided to the potential for bacterial adaptation, such as further induction of cross-resistance to other treatments and modifications in colonization or virulence (Dubois-Brissonnet, 2012). Detecting the mechanisms adopted by different bacteria to resist different drugs in various environmental niches remains important. In this respect, several studies report that physiological modifications occur during adaptation such as differential protein expression, which seems to be concomitant to increased tolerance (Dubois-Brissonnet, 2012) and cross-resistance to other environmental stressors (Karatzas et al., 2007, 2008).

In the last decade, proteomics has been used to study bacterial physiological responses to different stressors; this has progressed significantly with the availability of whole-genome sequences, progress in mass spectrometry and bioinformatics. Proteomics, as a key in the post genomic era, provides useful data to identify new diagnostic markers and therapeutic targets in diseases. Recently, genomic and proteomic analyses of *Lactobacillus* genus have rapidly expanded, especially with *Lb. pentosus* having one of the largest genomes known among LAB (Abriouel et al., 2011; Maldonado-Barragán et al., 2011); however, little is known about the mechanisms adopted by *Lb. pentosus* to tolerate or resist several stressors. This information should be of great concern since the knowledge of these mechanisms could be exploited to improve the functionality of probiotic starter strains and, thus, their health promoting benefits.

The present study aimed to determine the phenotypic and genotypic antimicrobial-resistance profiles of *Lb. pentosus* MP-10 and the selected mechanisms, by which these bacteria adapt under different antimicrobial stress. We compared the proteomic profiles of this strain induced by different antimicrobials (antibiotics or biocides), each with a distinct mechanism of action. The comparative analysis provides valuable knowledge and a broad overview of the key proteins involved in antibiotic and biocide tolerance.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Lb. pentosus MP-10, isolated from naturally-fermented Aloreña green table olives (Abriouel et al., 2011, 2012), was routinely cultured at 30 °C in Man Rogosa and Sharpe (MRS) broth (Fluka, Madrid, Spain) or agar under aerobic conditions for 24–48 h. The strain was stored long-term in 20% glycerol at –80 °C.

2.2. Antimicrobial agents

The antimicrobial agents used in this study were clinically relevant antibiotics: amoxicillin “AMX”, ampicillin “AMP”, cefuroxime “CFX”, chloramphenicol “CMP”, ciprofloxacin “CIP”, clindamycin “CLI”, erythromycin “ERY”, gentamicin “GEN”, kanamycin “KAN”, streptomycin “STR”, sulfamethoxazole/trimethoprim “SMZ/TMP”, teicoplanin “TC”, trimethoprim “TMP”, tetracycline “TET” and vancomycin “Van”; and biocides commonly used in the food industry: benzalkonium chloride “BC” and triclosan “TC”. All antibiotics and benzalkonium chloride were purchased from Sigma Aldrich (Madrid, Spain); however, triclosan was obtained from Fluka (Madrid, Spain).

2.3. Phenotypic and genotypic antibiotic testing

2.3.1. Antibiotic susceptibility testing and MIC determination

The MICs of the above-mentioned antibiotics were determined for *Lb. pentosus* MP-10 as described by Casado Muñoz et al. (2014) in LSM broth [a mixture of 90% IST broth (Oxoid, Madrid, Spain) and 10% MRS broth (Fluka, Madrid, Spain)] (Klare et al., 2005) according to the ISO 10932/IDF 233 standard (International Organization for Standardization, 2010).

2.3.2. PCR detection of antibiotic resistance genes

PCR amplifications of well-known gene determinants associated with resistance to β -lactam antibiotics (*bla* and *bla_Z*, the β -lactamase genes), sulfonamides (*dfrA* and *dfrD*) and glycopeptides (*vanA*, *vanB*, *vanC* and *vanE*) were performed using conditions described elsewhere (Dutka-Malen et al., 1995; Fines et al., 1999; Hummel et al., 2007; Liu et al., 2009; Martineau et al., 2000; Miele et al., 1995). Furthermore, PCR of genes mediating antibiotic resistance through other mechanisms, such as efflux pumps (*mdfA*, *norE*, *acrA*, *acrB*, *tolC*, *mepA*, *norA*, *norC*, *mefA* and *mdeA*), were also performed in the present study. Template DNA for PCR reactions were prepared as reported in Jensen et al. (1998).

2.4. Tolerance induction

Tolerance to antibiotics or biocides was assessed by investigating the ability of *Lb. pentosus* MP-10 to grow in the presence of sub-lethal concentrations of the corresponding antimicrobials, to which the strain was originally sensitive (amoxicillin, chloramphenicol, tetracycline, benzalkonium chloride and triclosan). Tolerant phenotypes were developed by increasing the concentrations of different antimicrobials as described by Casado Muñoz et al. (unpublished data). Briefly, antimicrobial tolerance in *Lb. pentosus* MP-10 was induced by exposure to triclosan (1 μ g/ml), benzalkonium chloride (1 μ g/ml), chloramphenicol (5 μ g/ml), tetracycline (10 μ g/ml) or amoxicillin (0.1 μ g/ml) at 30 °C for 48 h; cells were then harvested by centrifugation (Casado Muñoz et al., unpublished data). All *Lb. pentosus* isolates were stored in 20% glycerol at –80 °C until use. Isolates were streaked onto MRS-agar; a single colony was selected and subsequently used to inoculate MRS-broth for 24 h at 30 °C. The resulting culture was used to inoculate fresh MRS-broth at a dilution of 1:100. Cultures (both induced and non-induced controls) were harvested at mid-logarithmic growth phase ($OD_{600\text{ nm}} = 0.6$).

2.5. Whole cell protein extraction

The cell pellets obtained, as described above, from isogenic mutants were resuspended in 2 ml of PBS and dispersed into liquid nitrogen with a 200- μ l micropipette to obtain cryobeads. Whole-cell protein extraction was done as described by Caballero Gómez et al. (2013). The bacterial beads were ground in liquid nitrogen using a cryogenic grinder (6870 Freezer/Mill, SpexCertiPrep, Stanmore, UK) with three steps of 3 min at a rate of 24 impacts/s. The samples were centrifuged at 5000 $\times$ g for 5 min (at 4 °C), and the resultant supernatants were filtered through a 0.45- μ m pore size filter (Chromafil PET; Macherey-Nagel, Düren, Germany). Proteins were extracted from the filtered supernatants with TRIzol reagent (Euromedex, Souffelweyersheim, France) as previously described (Izquierdo et al., 2009). Protein concentrations were determined using the Bradford protein assay (Bio-Rad) according to the manufacturer's instructions.

2.6. 2-D gel electrophoresis

Protein extracts (150 μ g) were loaded onto 17-cm strips with a pH range of 3 to 10 (Bio-Rad), focused for 60,000 V h, and then separated on a 12% SDS-polyacrylamide gel as reported previously (Izquierdo

et al., 2009). The gels were stained as described by Candiano et al. (2004) using Bio-Safe Coomassie brilliant blue G-250 (Bio-Rad), which has a reported detection limit of 1 ng for BSA, and scanned on a GS-800 Calibrated Densitometer (Bio-Rad). Three independent experiments were carried out and each assay was performed in triplicate.

2.7. Image analysis

Image analysis of the 2D-GE gels was performed using PD Quest 8.0.1 software (Bio-Rad). Only spots that were present on the three gels were selected for inter-condition comparison. Spot intensities were normalized to the sum of intensities of all valid spots in one gel. For analysis of changes in protein expression during antimicrobial exposure, a protein was considered to be under- or over-produced when changes in normalized spot intensities were at least 1.5-fold at a significance level of $p < 0.05$ (Student's t test for paired samples), as previously described (Sánchez et al., 2007). Regarding proteome comparisons between different culture conditions of *Lb. pentosus* MP-10, proteins were considered differentially produced when spot intensities passed the threshold of a twofold difference (one-way ANOVA, p -value < 0.05), as described previously (Izquierdo et al., 2009).

2.8. Protein identification

Spots of interest were subjected to tryptic in-gel digestion as described by Izquierdo et al. (2009) and analyzed by chip-liquid chromatography–quadrupole time-of-flight (chip-LC–QTOF) using an Agilent G6510A QTOF mass spectrometer equipped with an Agilent 1200 Nano LC system and an Agilent HPLC Chip Cube, G4240A (Agilent Technologies, Santa Clara, CA, USA), as described previously (Hamon et al., 2011). Protein identification was performed against the genome of *Lb. pentosus* KCA1 available at the NCBI Website (<http://www.ncbi.nlm.nih.gov>; accessed 4th November 2014), using the PEAKS DB search engine (Bioinformatics Solutions Inc., Waterloo, Canada). Using the PEAKS inChorus feature, Mascot and PEAKS searches were compared to confirm protein identities and limit the risk of false positives. Scores represent peptide probabilities as calculated using PEAKS DB's Peptide-Spectrum Matching Score ($-10 \log p$).

2.9. Growth and survival of antimicrobial-induced and non-induced *Lb. pentosus* MP-10 following exposure to gastric juices

To determine the growth rate of antimicrobial-induced *Lb. pentosus* MP-10 in comparison with control (without induction), overnight cultures were diluted 1/1000 in MRS broth and viable counts were determined by serial dilutions on MRS-agar plates after 4 and 8 h of incubation at 30 °C. Increase in growth rate was determined by the difference between $\log_{10}$ CFU/ml at time $\times$ h (4 or 8 h) and $\log_{10}$ CFU/ml at time 0 h. To test if antimicrobial induction of *Lb. pentosus* MP-10 improved its tolerance to acid and bile concentrations, overnight cultures were added (at 2% volume) to simulated gastric juice (pepsin and NaCl) at different conditions: pH 1.5, pH 2.5, 2% bile or 3% bile. The mixtures were incubated at 37 °C for 30 min and viable counts were determined on MRS agar plates as described above. The survival rate was determined according to Bao et al. (2010) by the following equation: survival rate (%) = $(\log_{10} \text{ CFU/ml } N_1 / \log_{10} \text{ CFU/ml } N_0) \times 100$.

N_1 is the total viable count of *Lb. pentosus* MP-10 after 30 min treatment (at pH 1.5, pH 2.5, 2% bile or 3% bile), and N_0 is the total viable count at time 0 (before treatment).

3. Results

3.1. Antibiotic susceptibility and molecular detection of antibiotic resistance genes in *Lb. pentosus* MP-10

MIC determinations of the different antibiotics revealed that *Lb. pentosus* MP-10 was sensitive to amoxicillin (MIC = 0.2 µg/ml), ampicillin (MIC = 0.2 µg/ml), chloramphenicol (MIC = 0.04 µg/ml), clindamycin (MIC = 0.2 µg/ml), erythromycin (MIC = 0.1 µg/ml), gentamycin (MIC = 0.8 µg/ml), kanamycin (MIC = 16 µg/ml), streptomycin (MIC = 150 µg/ml) and tetracycline (MIC = 8 µg/ml). However, *Lb. pentosus* MP-10 showed resistance to cefuroxime (MIC = 100 µg/ml), ciprofloxacin (MIC = 8 µg/ml), teicoplanin (MIC > 128 µg/ml), trimethoprim (MIC = 128 µg/ml), trimethoprim/sulfamethoxazole (MIC = 950/50 µg/ml) and vancomycin (MIC > 128 µg/ml). In most cases, resistance or sensitivity was categorized based on the microbiological breakpoints of the antibiotics tested (also defined as ECOFF by the European Food Safety Authority, 2012), which was reviewed by Casado Muñoz et al. (2014).

To identify possible genetic determinants responsible for the resistance phenotypes observed in *Lb. pentosus* MP-10, PCR reactions were performed as described above. However, results revealed an absence of specific resistance determinants, except for the detection of *norA* coding for a multidrug efflux pump.

3.2. Influence of antibiotics on protein expression levels in *Lb. pentosus* MP-10

Based on antibiotic susceptibility results, amoxicillin, chloramphenicol and tetracycline were selected to carry out tolerance studies. We compared the proteomes of antibiotic-treated and untreated *Lb. pentosus* MP-10 to elucidate the physiological changes resulting from the treatments. 2D-GE analysis of antibiotic-treated cells, collected during mid-exponential growth phase, showed different proteomic profiles depending on the antibiotic used, suggesting various antibiotic stress responses (Fig. 1). Treatment with chloramphenicol, amoxicillin and tetracycline resulted in two, four and six proteins (respectively) that significantly ($p < 0.05$) differed to the pattern from the untreated control (Fig. 1). These proteins were individually excised from duplicate 2D-GE gels, subjected to tryptic digestion, and identified by chip-LC-QTOF and Uniprot database searching (summarized in Table 1). Treatment with amoxicillin or chloramphenicol resulted in an under-expressed CTP synthase (spot 0102), an enzyme involved in nucleotide synthesis that requires ATP for its metabolic function. On the other hand, proteins involved in other metabolic pathways such as carbohydrate metabolism (phosphocarrier protein HPr of the phosphotransferase system "PTS", spot 4201), homeostasis (NADH peroxidase Npx, spot 6101) and protein synthesis (SSU ribosomal protein S6p, spot 7202) became over-expressed in the presence of amoxicillin. Similarly, three proteins carrying different biological functions were over-expressed in the presence of tetracycline: 6-phosphogluconate dehydrogenase (spot 7605), involved in carbohydrate metabolism; a small heat shock protein (spot 7802) responsible of cell protection; and LSU ribosomal protein L1p (spot 7803), implicated in protein synthesis (Fig. 1, Table 1).

The following three proteins were only expressed in the absence of tetracycline: pyruvate kinase (spot 3102) and NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (spot 4501), which are linked to carbohydrate metabolism, as well as acetaldehyde dehydrogenase (spot 3104), which is involved in alcohol and fat metabolism (Fig. 1, Table 1). In the case of chloramphenicol, the only protein not produced, compared with the untreated control, was 6-phosphofructokinase, which is related to carbohydrate metabolism.

Fig. 1. 2-DE gels of whole cell proteomes from *Lactobacillus pentosus* MP-10 cultured in the absence (A) or presence of amoxicillin (B), chloramphenicol (C) and tetracycline (D). The figure shows representative 2-DE gel pictures (pH range: 4–7) of whole-cell protein lysates from an early stationary phase of *Lb. pentosus* MP-10 spots exhibiting constitutive differential expression between growth of *Lb. pentosus* MP-10 in standard conditions and after induction by antibiotics which were identified by peptide mass fingerprinting and are labeled; the identifications of the spots affected by antibiotics are listed in Table 1.

3.3. Influence of biocides on protein expression levels in *Lb. pentosus* MP-10

According to biocide susceptibility pattern by *Lb. pentosus* MP-10 (Casado Muñoz et al., unpublished data), we selected benzalkonium chloride and triclosan for further tolerance studies. Following treatment with biocides (benzalkonium chloride or triclosan), the proteomes of *Lb. pentosus* MP-10 were compared with untreated bacteria. The benzalkonium chloride exposure resulted in only one protein significantly ($p < 0.05$) over-expressed in the induced cells: ribosomal subunit interface protein (spot 6603), which is related to protein biosynthesis (Fig. 2, Table 2). However, the proteome of *Lb. pentosus* MP-10 treated with triclosan showed significant ($p < 0.05$) differential expression among three proteins: an over-expressed glutamyl-tRNA synthetase (spot 5801), linked to amino acid starvation; an under-expressed phosphocarrier protein HPr of the PTS (spot 4401), related to carbohydrate metabolism; and no detection of oxidoreductase of the aldo/keto reductase family (spot 5301), involved in energy production and conversion (Fig. 2, Table 2).

3.4. Survival and tolerance responses of antimicrobial-induced *Lb. pentosus* MP-10

As shown in Table 3, the growth rate was increased in almost all antimicrobial-induced *Lb. pentosus* MP-10 by 0.09–0.32 Log₁₀ units after 4 or 8 h incubation at 30 °C except in chloramphenicol-induced cells, which showed the same growth rate as non-induced controls.

Comparison of survival capacity of non-induced and antimicrobial-induced *Lb. pentosus* MP-10 under acid or bile (2 and 3%) stress determined that antimicrobial induction improved the tolerance capacity of *Lb. pentosus* MP-10 at acidic conditions. The bacteria had > 100% survival and they exhibited slightly greater growth than the controls (94% and 100%, at pH 1.5 and 2.5, respectively) (Table 3). Regarding bile tolerance, at both concentrations of 2 and 3% of bile we observed 100% survival, or better, in benzalkonium- and triclosan-induced cells; moreover at 2% bile concentration, chloramphenicol-induced *Lb. pentosus* MP-10 also showed 100% survival (Table 3). However, at 3% bile

Table 1

Proteins differentially expressed in *Lactobacillus pentosus* MP-10 treated with different antibiotics, as compared to control.

Protein identity	Spot no.	Accession number ^a	– 10 lgP ^b	Sequence coverage (%)
Proteins over-expressed				
In the presence of amoxicillin:				
Phosphotransferase system, phosphocarrier protein HPr	4201	EIW14262.1	341.26	97
NADH peroxidase Npx (EC 1.11.1.1)	6101	EIW13302.1	372.87	78
SSU ribosomal protein S6p	7202	EIW15333.1	240.77	87
In the presence of tetracycline:				
6-phosphogluconate dehydrogenase, decarboxylating (EC 1.1.1.44)	7605	EIW14063.1	407.61	82
Small heat shock protein	7802	EIW15192.1	315.52	97
LSU ribosomal protein L1p	7803	EIW14839.1	333.50	66
Protein under-expressed				
In the presence of amoxicillin or chloramphenicol:				
CTP synthase (EC 6.3.4.2)	0102	EIW15026.1	384.76	75
Proteins not expressed				
In the presence of chloramphenicol:				
6-Phosphofructokinase (EC 2.7.1.11)	5501	EIW13706.1	358.39	88
In the presence of tetracycline:				
Pyruvate kinase (EC 2.7.1.40)	3102	EIW13705.1	381.86	94
Alcohol dehydrogenase (EC 1.1.1.1); acetaldehyde dehydrogenase (EC 1.2.1.10)	3104	EIW15075.1	317.41	63
NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	4501	EIW14737.1	411.19	91

^a Accession number in the NCBI database.

^b PEAKS DB's Peptide-Spectrum Matching Score.

Table 2

Proteins differentially expressed in *Lactobacillus pentosus* MP-10 treated with different biocides as compared to control.

Protein identity	Spot no.	Accession number ^a	– 10 lgP ^b	Sequence coverage (%)
Proteins over-expressed				
In the presence of benzalkonium chloride:				
Ribosomal subunit interface protein	6603	EIW14771.1	310.89	67
In the presence of triclosan:				
Glutamyl-tRNA synthetase (EC 6.1.1.17)	5801	EIW14887.1	334.84	70
Protein under-expressed				
In the presence of triclosan:				
Phosphotransferase system, phosphocarrier protein HPr	4401	EIW14262.1	181.75	57
Proteins not expressed				
In the presence of triclosan:		EIW12654.1		
Oxidoreductase of aldo/keto reductase family, subgroup 1	5301		322.88	73

^a Accession number in the NCBI database.

^b PEAKS DB's Peptide-Spectrum Matching Score.

concentration, bacteria pre-exposed to amoxicillin, chloramphenicol and tetracycline became less viable (Table 3).

4. Discussion

The importance of probiotic bacteria, which are mainly members of the genera *Lactobacillus* and *Bifidobacterium*, has increasingly become recognized in human and animal nutrition by their contributions to immunological, digestive, and respiratory health. However, according to

the Qualified Presumption of Safety (QPS) approach proposed by the European Food Safety Authority (EFSA, 2008), the presence of antibiotic resistance determinants is one of the most important safety selection criteria for bacterial strains intended for use in the food industry, even among bacteria that are generally recognized as “safe”. Here, *Lb. pentosus* MP-10 isolated from brines of naturally fermented Aloreña green table olives (Abriouel et al., 2011, 2012) could be regarded as “safe” because of the absence of acquired resistance determinants. Their intrinsic resistance to more than three antibiotics, which relies on chromosomally encoded efflux pumps such as NorA, is unlikely to be an issue from a medical point of view, since *Lb. pentosus* MP-10 remains highly sensitive to other clinically relevant antibiotics.

However, the survival of probiotic bacteria and their beneficial probiotic effects under different environmental conditions, including those encountered in the gastrointestinal tract, may rely on the resistance traits. As such, knowing which proteins are involved in tolerance is important to improve the functionality of probiotic strains under different stress conditions. In the present study, we investigated the proteomic response of probiotic bacteria *Lb. pentosus* MP-10 to antimicrobial stress conditions. Antibiotics and biocides induced adaptations in *Lb. pentosus*

Fig. 2. 2-DE gels of whole cell proteomes from *Lactobacillus pentosus* MP-10 cultured in the absence (A) or presence of benzalkonium chloride (B) and triclosan (C). The figure shows representative 2-DE gel pictures (pH range: 4–7) of whole-cell protein lysates from an early stationary phase of *Lb. pentosus* MP-10 spots exhibiting constitutive differential expression between growth of *Lb. pentosus* MP-10 in standard conditions and after induction by biocides which were identified by peptide mass fingerprinting and are labeled; the identifications of the spots affected by biocides are listed in Table 2.

Table 3Growth and survival of the antimicrobial-induced *Lactobacillus pentosus* MP-10 after exposition to acidic pH and bile concentrations.

Antimicrobial induced- <i>Lactobacillus pentosus</i> MP-10	Increase in growth rate (Log ₁₀ CFU/ml) ^a		Survival to acidic pH during 30 min (%)		Survival to bile concentration during 30 min (%)	
	4 h	8 h	pH 1.5	pH 2.5	2%	3%
Control (without induction)	0.45 ± 0.17	1.35 ± 0.18	94	100	67	87.5
Benzalkonium chloride	0.76 ± 0.06	1.43 ± 0.07	>100	>100	100	100
Triclosan	0.47 ± 0.24	1.24 ± 0.05	>100	>100	>100	>100
Amoxicillin	0.39 ± 0.06	1.44 ± 0.03	>100	>100	71	29
Chloramphenicol	0.43 ± 0.01	1.34 ± 0.10	>100	>100	>100	40
Tetracycline	0.59 ± 0.05	1.2 ± 0.01	>100	>100	60	64

Each value or percentage in the table represents the mean of triplicate plate count readings from three separate experiments.

^a Difference between Log₁₀ CFU/ml at different times (4 or 8 h) and Log₁₀ CFU/ml at time 0.

MP-10 as evidenced by modifications of its proteomic arsenal, with the observed changes being intimately dependent on the antimicrobial used. Overall, antibiotics induced several physiological modifications, possibly due to various mechanisms of action, each targeting a defined cellular structure; in comparison, biocides induced fewer modifications. Adaptation to antibiotics is likely to trigger comparatively more physiological modifications than biocides; several resistance mechanisms to antibiotics have had a longer evolution process to protect bacteria, compared with the more relatively recent exposure to biocides and limited opportunity to develop resistance. Overall, several proteins involved in carbohydrate metabolism like phosphocarrier protein HPr of the PTS, as part of glycolysis-related machinery, and 6-phosphogluconate dehydrogenase of the pentose phosphate pathway were up-regulated after exposure to antibiotics (amoxicillin or tetracycline) targeting different cellular structures. Increasing the level of ATP synthesis (Wilkins et al., 2002) was either required for the increased efflux activity or compensating the low glycolytic capacity (Wouters et al., 2000), and is an important factor for survival under stress conditions. Similar results were obtained with *Bifidobacterium animalis* and *Lactobacillus reuteri* under bile stress (Lee et al., 2008; Sánchez et al., 2007). Furthermore, HPr (histidine-containing protein) protein is not only responsible for carbohydrate uptake; it also plays a regulatory role in sugar metabolism and catabolite repression, depending on protein–protein interactions with many cellular factors (Deutscher et al., 2006). Accordingly, other proteins involved in glycolysis pathways such as 6-phosphofructokinase, and pyruvate kinase and NAD-dependent glyceraldehyde-3-phosphate dehydrogenase were down-regulated in the presence of antibiotics inhibiting protein synthesis – chloramphenicol and tetracycline, respectively. Pyruvate, an end product of glycolysis, is a metabolic key molecule that can be used in a number of different reactions to increase the ATP levels, thus antibiotic stress induced regulation of metabolism by down- or up-regulation of enzymes involved in energy production. These data suggest that, to ensure survival under antibiotic stress, *Lb. pentosus* MP-10 physiology may be altered to achieve a higher cellular energy gain via up- or down-regulation of carbohydrate metabolism (pentose and glycolysis pathways). Under antibiotic stress and subsequent limited energy conditions, PTS transport systems are used rather than ABC transporters (Taranto et al., 1999). These systems are, in fact, more energy efficient as the phosphorylated substrate can directly enter glycolysis or pentose phosphate pathways, conserving ATP. Similarly, Lin et al. (2014) reported that fluctuation of metabolic pathways may represent an antibiotic-resistance mechanism under chlortetracycline stress in *Escherichia coli*.

The interaction of amoxicillin and tetracycline with membrane lipids and proteins induced the over-expression of stress proteins, such as NADH peroxidase Npx and a small heat shock protein, respectively, as a first response of the cell to maintain homeostasis and viability. Furthermore, it has been reported that, besides its role in cell redox homeostasis (degradation of hydrogen peroxide to water and oxygen), Npx of the peroxidase–oxidase–reductase (POR) subgroup of the flavoprotein–disulfide–reductase (FDR) family also contributes to the

regeneration of oxidized pyridine nucleotides for glycolysis (Ying, 2006). Small heat shock proteins as “minichaperones” have been associated with enhanced bacterial survival during stress, since they are necessary for normal cellular functions, including growth and stability of DNA and RNA. They also prevent the formation of inclusion bodies (Jakob et al., 1993; Narberhaus, 2002; Veinger et al., 1998), but are not involved in protein re-folding as chaperones.

On the other hand, protein synthesis in *Lb. pentosus* MP-10, exposed to amoxicillin and tetracycline, was up-regulated. However, it has been reported that the proteins involved in cell growth, such as ribosomal proteins, were markedly under-regulated under stress conditions as an energy-saving strategy necessary for protection mechanisms in the cell (Rezzonico et al., 2007). In spite of the fact that ribosomal run-off and transit times are slower upon stressor exposure, stress-regulatory factors are preferentially associated with ribosomes, suggesting increased translation and protein synthesis (Sherman and Qian, 2013). Enhanced protein synthesis may be required to compensate for the proteins damaged as a result of the interaction of antibiotics with the membrane or cytoplasmic proteins, regardless of their cellular target. Some may be involved in metabolism or defense (SOS response and heat shock response). Similarly, Mangalappalli-Illathu and Korber (2006) reported that higher levels of ribosomal proteins associated with increased protein synthesis were important for reduced susceptibility to quaternary ammonium compounds like benzalkonium chloride.

Concerning other metabolic pathways, the enzymes involved in fatty-acid (alcohol dehydrogenase) and pyrimidine biosynthesis (CTP synthase) were down-regulated in the presence of antibiotics. Alterations in fatty-acid biosynthesis may lead to changes in the cell membrane that would favor cell survival in the presence of tetracycline; Rogers et al. (2007) obtained similar results with penicillin-exposed *Streptococcus pneumoniae*. Regarding CTP synthase, this enzyme is required for the biosynthesis of ribo- and deoxyribonucleotides for RNA and DNA replication (Jørgensen et al., 2004). Lowered growth rates obtained just after exposure to antibiotics may have reflected the down-regulation of proteins involved in nucleotide synthesis and fatty acids. However, after antimicrobial exposure, growth rates were either similar or even increased in some antimicrobial-induced cells (e.g., amoxicillin- or benzalkonium-induced cells).

On the other hand, the adaptation of *Lb. pentosus* MP-10 to biocides (benzalkonium chloride or triclosan) induced physiological modifications that are, in part, similar to those caused by antibiotics such as up-regulation of protein synthesis, and down-regulation of carbohydrate metabolism and energy production (Fig. 3). In fact, cross-resistance between antibiotics and biocides was widely reported in literature (e.g., Fraiese, 2002; Moken et al., 1997; Randall et al., 2007). Also, in a previous study, *Lb. pentosus* MP-10 pre-adapted to low concentrations of biocides showed increased antibiotic MICs (Casado Muñoz et al., unpublished data), suggesting that the physiological modifications triggered by either a biocide or an antibiotic may provide resistance to the other. Benzalkonium chloride, a disinfectant known to cause membrane damage, specifically induced an over-expression

Fig. 3. Schematic representation of the effect of antibiotics and biocides on protein expression in *Lactobacillus pentosus* MP-10.

of ribosomal subunit interface protein related to protein synthesis. However, triclosan caused over-expression of glutamyl-tRNA synthetase, which is considered a key enzyme required for protein biosynthesis. Furthermore, triclosan caused down-regulation of proteins involved in carbohydrate metabolism (phosphocarrier protein HPr) and energy production (oxidoreductase). As stated previously with antibiotics, cells adapted to antimicrobials tended to lower carbohydrate metabolism and energy production, while those involved in protein synthesis were up-regulated to possibly compensate for protein damage as a result of the interaction of biocides with the membrane. Moreover, benzalkonium chloride and triclosan exhibited different adaptation responses, which may be attributed to different mechanisms of action; triclosan acts by inhibiting the enoyl reductase enzyme in fatty acid synthesis (Heath et al., 2002), while benzalkonium chloride has multiple targets in microbial cells (Beumer et al., 2000).

In conclusion, we obtained a better understanding of the proteomic responses of a probiotic bacterium, such as *Lb. pentosus* MP-10 to different antimicrobial stressors. In this sense, we confirmed that antimicrobial stress could enhance bacterial resistance to environmental and gastrointestinal stresses such as acid and bile. Thus, viable counts of some antimicrobial-induced *Lb. pentosus* MP-10 were higher than the non-induced strain. From this information, one could develop strategies to improve the persistence and resistance of this bacterium under different environmental conditions. It has been previously shown that adaptation to different stresses (salt, low pH, bile, high temperature, etc.) could be used as a strategy to enhance the technological performance of probiotic lactobacilli (Corcoran et al., 2006; Desmond et al., 2001; Mills et al., 2011). In our study, pre-stressed *Lb. pentosus* MP-10 exhibited greater viability than those without previous induction (except for a few cases) and had increased tolerance to acidic and high-bile environments than the controls. Here, we describe for the first time that antimicrobial stress adaptation could improve the resistance and robustness of potential probiotic *Lb. pentosus* MP-10 with the aim to withstand

conditions where sub-lethal concentrations of antimicrobials and stress conditions (e.g., at low pH or high-bile concentration) may be present, such as the food chain, the environment, or the gastrointestinal tract. On the other hand, this fact has become a great concern since pathogenic bacteria, as they can develop antimicrobial resistance after exposure to antimicrobials, could possibly develop resistance to intestinal conditions. Our results show that *Lb. pentosus* MP-10 responds to the exposure of biocides and antibiotics by adjusting its proteomic arsenal as a survival strategy: up-regulating protein synthesis, including stress proteins, and down-regulating carbohydrate metabolism and energy production (Fig. 3). Further, studies are required to elucidate which proteins are involved in acid and bile tolerance. These aspects should be further emphasized with the aim to achieve desirable robustness of probiotic bacteria in relation to various environmental and gastrointestinal conditions.

Acknowledgments

This work was supported by grants AGL2013-43571-P (Ministerio de Economía y Competitividad, MINECO, FEDER), AGL2009-08921, (Ministerio de Economía y Competitividad, MINECO) and UJA2014/07/02 (Plan Propio de la Universidad de Jaén).

References

- Abriouel, H., Benomar, N., Pulido, R.P., Cañamero, M.M., Gálvez, A., 2011. Annotated genome sequence of *Lactobacillus pentosus* MP-10, which has probiotic potential, from naturally fermented Aloreña green table olives. *J. Bacteriol.* 193, 4559–4560.
- Abriouel, H., Benomar, N., Cobo, A., Caballero, N., Fernández Fuentes, M.A., Pérez-Pulido, R., Gálvez, A., 2012. Characterization of lactic acid bacteria from naturally-fermented Manzanilla Aloreña green table olives. *Food Microbiol.* 32, 308–316.
- Anukam, K.C., Olise, N.A., 2012. Development of a novel probiotic yogurt “PENTYOY” with a fully sequenced *Lactobacillus pentosus* KCA1 and its survival during storage at 4 °C. *Trop. J. Pharm. Res.* 11, 977–981.
- Anukam, K.C., MacLaim, J.M., Gloor, G.B., Reid, G., Boekhorst, J., Renckens, B., van Hijum, S., Siezen, R., 2013. Genome sequence of *Lactobacillus pentosus* KCA-1 vaginal isolate from a healthy premenopausal woman. *PLoS One* 8, e59239.
- Bao, Y., Zhang, Y.C., Zhang, Y., Liu, Y., Wang, S.Q., Dong, X.M., Wang, Y.Y., Zhang, H.P., 2010. Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. *Food Control* 21, 695–701.
- Bautista-Gallego, J., Arroyo-López, F.N., Rantsiou, K., Jiménez-Díaz, R., Garrido-Fernández, A., Cocolin, L., 2013. Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. *Food Res. Int.* 50, 135–142.
- Beumer, R., Bloomfield, S.F., Exner, M., Fara, G.M., Nath, K.J., Scott, E., 2000. Microbial resistance and biocides. A Review by the International Scientific Forum on Home Hygiene. International Scientific Forum on Home Hygiene, London, UK (<http://www.iffh-homehygiene.org/IntegratedCRD.nsf/111e68ea0824afe1802575070003f039/f309bd6a403617fa80257522005bc5f9?OpenDocument>).
- Caballero Gómez, N., Abriouel, H., Ennahar, S., Gálvez, A., 2013. Comparative proteomic analysis of *Listeria monocytogenes* exposed to enterocin AS-48 in planktonic and sessile states. *Int. J. Food Microbiol.* 167, 202–207.
- Candiano, G., Bruschi, M., Musante, L., Sabntucci, L., Ghiggeri, G.M., Carnemolla, B., Orecchia, P., Zardi, L., Righetti, P.G., 2004. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis* 25, 1327–1333.
- Casado Muñoz, M.C., Benomar, N., Lavilla Lerma, L., Gálvez, A., Abriouel, H., 2014. Antibiotic resistance of *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* isolated from naturally-fermented Aloreña table olives throughout fermentation process. *Int. J. Food Microbiol.* 172, 110–118.
- Corcoran, B.M., Ross, R.P., Fitzgerald, G.F., Dockery, P., Stanton, C., 2006. Enhanced survival of GroESL-overproducing *Lactobacillus paracasei* NFBC 338 under stressful conditions induced by drying. *Appl. Environ. Microbiol.* 72, 5104–5107.
- Desmond, C., Stanton, C., Fitzgerald, G.F., Collins, K., Ross, R.P., 2001. Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying. *Int. Dairy J.* 11, 801–808.
- Deutscher, J., Francke, C., Postma, P.W., 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol. Mol. Biol. Rev.* 70, 939–1031.
- Dubois-Brissonnet, F., 2012. Adaptation of *Salmonella* to antimicrobials in food-processing environments. In: Annous, B.A., Gurtler, J.B. (Eds.), *Agricultural and Biological Sciences “Salmonella – Distribution, Adaptation, Control Measures and Molecular Technologies”*. Intech, pp. 123–146.
- Duran, G.M., Marshall, D.L., 2005. Ready-to-eat shrimp as an international vehicle of antibiotic-resistant bacteria. *J. Food Prot.* 68, 2395–2401.
- Dutka-Malen, S., Stefan, E., Courvalin, P., 1995. Detection of glycopeptides resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J. Clin. Microbiol.* 33, 24–27.
- European Food Safety Authority (EFSA), 2008. Technical guidance prepared by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the

- update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. EFSA J. 732, 1–15.
- EFSA Panel on Biological Hazards (BIOHAZ), 2010. Scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2010 update). EFSA J. 8, 1944.
- European Food Safety Authority (EFSA), 2011. Scientific opinion on the safety and efficacy of *Lactobacillus pentosus* (DSM 14025) as a silage additive for all animal species. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). EFSA J. 9, 2449.
- European Food Safety Authority (EFSA), 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J. 10, 2740.
- Fines, M., Perichon, B., Reynolds, P., Sahm, D.F., Courvalin, P., 1999. VanE, a new type of acquired glycopeptide resistance in *Enterococcus faecalis* BM4405. Antimicrob. Agents Chemother. 43, 2161–2164.
- Fraise, A.P., 2002. Susceptibility of antibiotic-resistant cocci to biocides. J. Appl. Microbiol. 92, 158S–162S.
- Franz, C.M.A.P., Holzapfel, W.H., Stiles, M.E., 1999. Enterococci at the crossroad of food safety. Int. J. Food Microbiol. 47, 1–24.
- Hamon, E., Horvatovich, P., Izquierdo, E., Bringel, F., Marchioni, E., Aoude-Werner, D., Ennahar, S., 2011. Comparative proteomic analysis of *Lactobacillus plantarum* or the identification of key proteins in bile tolerance. BMC Microbiol. 11, 63.
- Heath, R.J., White, S.W., Rock, C.O., 2002. Inhibitors of fatty acid synthesis as antimicrobial chemotherapeutics. Appl. Microbiol. Biotechnol. 58, 695–703.
- Hummel, A.S., Hertel, C., Holzapfel, W.H., Franz, C.M.A.P., 2007. Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. Appl. Environ. Microbiol. 73, 730–739.
- International Organization for Standardization (ISO), 2010. Standard milk and milk products—determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB). ISO 10932/IDF 233. ISO, Geneva, Switzerland.
- Izquierdo, E., Horvatovich, P., Marchioni, E., Aoude-Werner, D., Sanz, Y., Ennahar, S., 2009. 2-DE and MS analysis of key proteins in the adhesion of *Lactobacillus plantarum*, a first step toward early selection of probiotics based on bacterial biomarkers. Electrophoresis 30, 949–956.
- Jakob, U., Gaestel, M., Engel, K., Buchner, J., 1993. Small heat shock proteins are molecular chaperones. J. Biol. Chem. 268, 1517–1520.
- Jensen, L.B., Ahrens, P., Dons, L., Jones, R.N., Hammerum, A.M., Aarestrup, F.M., 1998. Molecular analysis of Tn1546 in *Enterococcus faecium* isolates from animals and humans. J. Clin. Microbiol. 36, 437–442.
- Jørgensen, C.M., Hammer, K., Jensen, P.R., Martinussen, J., 2004. Expression of the *pyrG* gene determines the pool sizes of CTP and dCTP in *Lactococcus lactis*. Eur. J. Biochem. 271, 2438–2445.
- Karatzas, K.A.G., Webber, M.A., Jørgensen, F., Woodward, M.J., Piddock, L.J.V., Humphrey, T.J., 2007. Prolonged treatment of *Salmonella enterica* serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. J. Antimicrob. Chemother. 60, 947–955.
- Karatzas, K.A.G., Randall, L.P., Webber, M., Piddock, L.J.V., Humphrey, T.J., Woodward, M.J., Coldham, N.G., 2008. Phenotypic and proteomic characterization of multiply antibiotic-resistant variants of *Salmonella enterica* serovar Typhimurium selected following exposure to disinfectants. Appl. Environ. Microbiol. 74, 1508–1516.
- Klare, I., Konstabel, C., Müller-Bertling, S., Reissbrodt, R., Huys, G., Vancanneyt, M., Swings, J., Goossens, H., Witte, W., 2005. Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. Appl. Environ. Microbiol. 71, 8982–8986.
- Lee, K., Lee, H.G., Choi, Y.J., 2008. Proteomic analysis of the effect of bile salts on the intestinal and probiotic bacterium *Lactobacillus reuteri*. J. Biotechnol. 137, 14–19.
- Lin, X., Kang, L., Li, H., Peng, X., 2014. Fluctuation of multiple metabolic pathways is required for *Escherichia coli* in response to chlortetracycline stress. Mol. Biosyst. 10, 901–908.
- Liu, C., Zhang, Z., Dong, K., Yuan, J., Guop, X., 2009. Antibiotic resistance of probiotic strains of lactic acid bacteria isolated from marketed foods and drugs. Biomed. Environ. Sci. 22, 401–412.
- Maisonneuve, E., Gerdes, K., 2014. Molecular mechanisms underlying bacterial persisters. Cell 157, 539–548.
- Maldonado-Barragán, A., Caballero-Guerrero, B., Lucena-Padrós, H., Ruiz-Barba, J.L., 2011. Genome sequence of *Lactobacillus pentosus* IG1, a strain isolated from Spanish-style green olive fermentations. J. Bacteriol. 193, 5605.
- Mangalappalli-Illathu, A.K., Korber, D.R., 2006. Adaptive resistance and differential protein expression of *Salmonella enterica* serovar Enteritidis biofilms exposed to benzalkonium chloride. Antimicrob. Agents Chemother. 50, 3588–3596.
- Martineau, F., Picard, F.J., Grenier, L., Roy, P.H., Ouellette, M., Bergeron, M.G., 2000. Multiplex PCR assays for the detection of clinically relevant antibiotic resistance genes in staphylococci isolated from patients infected after cardiac surgery. J. Antimicrob. Chemother. 46, 527–533.
- Miele, A., Bandera, M., Goldstein, B.P., 1995. Use of primers selective for vancomycin resistance genes to determine van genotype in enterococci and to study gene organization in VanA isolates. Antimicrob. Agents Chemother. 39, 1772–1778.
- Mills, S., Stanton, C., Fitzgerald, G.F., Ross, R.P., 2011. Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. Microb. Cell Fact. 10 (Suppl. 1), S19.
- Moken, M.C., McMurry, L.M., Levy, S.B., 1997. Selection of multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli* by using the disinfectant pine oil: roles of the *mar* and *acrAB* loci. Antimicrob. Agents Chemother. 41, 2770–2772.
- Narberhaus, F., 2002. Alpha-crystallin-type heat shock proteins: socializing minichaperones in the context of a multichaperone network. Microbiol. Mol. Biol. Rev. 66, 64–93.
- Okada, S., Daengsubha, W., Uchimura, T., Ohara, N., Kozaki, M., 1986. Flora of lactic acid bacteria in Miang produced in Northern Thailand. J. Gen. Appl. Microbiol. 32, 57–65.
- Randall, L.P., Cooles, S.W., Coldham, N.G., Penuela, E.G., Mott, A.C., Woodward, M.J., Piddock, L.J.V., Webber, M.A., 2007. Commonly used farm disinfectants can select for mutant *Salmonella enterica* serovar Typhimurium with decreased susceptibility to biocides and antibiotics without compromising virulence. J. Antimicrob. Chemother. 60, 1273–1280.
- Rezzonico, E., Lariani, S., Barretto, C., Cuanoud, G., Giliberti, G., Delley, M., Arigoni, F., Pessi, G., 2007. Global transcriptome analysis of the heat shock response of *Bifidobacterium longum*. FEMS Microbiol. Lett. 271, 136–145.
- Rodríguez-Gómez, F., Romero-Gil, V., García-García, P., Garrido-Fernández, A., Arroyo-López, F.N., 2014. Fortification of table olive packing with the potential probiotic bacteria *Lactobacillus pentosus* TOMC-LAB2. Front. Microbiol. 5, 467.
- Rogers, P.D., Liu, T.T., Barker, K.S., Hilliard, G.M., English, B.K., Thornton, J., Swiatlo, E., McDaniel, L.S., 2007. Gene expression profiling of the response of *Streptococcus pneumoniae* to penicillin. J. Antimicrob. Chemother. 59, 616–626.
- Ruiz-Barba, J.L., Jiménez-Díaz, R., 2012. A novel *Lactobacillus pentosus*-paired starter culture for Spanish-style green olive fermentation. Food Microbiol. 30, 253–259.
- Sánchez, B., Champomier-Vergès, M.C., Stuer-Lauridsen, B., Ruas-Madiedo, P., Anglade, P., Baraige, F., delos Reyes-Gavilán, C.G., Johansen, E., Zagorec, M., Margolles, A., 2007. Adaptation and response of *Bifidobacterium animalis* subsp. *lactis* to bile: a proteomic and physiological approach. Appl. Environ. Microbiol. 73, 6757–6767.
- Sherman, M.Y., Qian, S.B., 2013. Less is more: improving proteostasis by translation slow down. Trends Biochem. Sci. 38, 585–591.
- Tajabadi, N., Mardan, M., Abdul Manap, M.Y., Shuhaimi, M., Meimandipour, A., Nateghi, L., 2011. Detection and identification of *Lactobacillus* bacteria found in the honey stomach of the giant honeybee *Apis dorsata*. Apidologie 42, 642–649.
- Taranto, M.P., Font de Valdez, G., Perez-Martinez, G., 1999. Evidence of a glucose proton motive force-dependent permease and a fructose phosphoenolpyruvate:phosphotransferase transport system in *Lactobacillus reuteri* CRL 1098. FEMS Microbiol. Lett. 181, 109–112.
- Todorov, S.D., Dicks, L.M.T., 2004. Effect of medium components on bacteriocin production by *Lactobacillus pentosus* ST151BR, a strain isolated from beer produced by the fermentation of maize, barley and soy flour. World J. Microbiol. Biotechnol. 20, 643–650.
- Veinger, L., Diamant, S., Buchner, J., Goloubinoff, P., 1998. The small heat-shock protein IbpB from *Escherichia coli* stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. J. Biol. Chem. 273, 11032–11037.
- Wilkins, J.C., Homer, K.A., Beighton, D., 2002. Analysis of *Streptococcus mutans* proteins modulated by culture under acidic conditions. Appl. Environ. Microbiol. 68, 2382–2390.
- Wouters, J.A., Kamphuis, H.H., Hugenholtz, J., Kuipers, O.P., De Vos, W.M., Abee, T., 2000. Changes in glycolytic activity of *Lactococcus lactis* induced by low temperature. Appl. Environ. Microbiol. 66, 3686–3691.
- Ying, W., 2006. NAD⁺ and NADH in cellular functions and cell death. Front. Biosci. 11, 3129–3148.
- Zhang, X.X., Zhang, T., Fang, H.H., 2009. Antibiotic resistance genes in water environment. Appl. Microbiol. Biotechnol. 82, 397–414.